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Expression and purification of a trivalent pertussis toxin–diphtheria toxin–tetanus toxin fusion protein in *Escherichia coli*

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Abstract

Pertussis toxoid, diphtheria toxoid, and tetanus toxoid are key components of diphtheria–tetanus–acellular pertussis vaccines. The efficacy of the vaccines is well documented, however, the vaccines are expensive partly because the antigens are derived from three different bacteria. In this study, a fusion protein (PDT) composed of the immunoprotective S1 fragment of pertussis toxin, the full-length non-toxic diphtheria toxin, and fragment C of tetanus toxin was constructed via genetic means. The correct fusion was verified by restriction endonuclease analysis and Western immunoblotting. *Escherichia coli* carrying the recombinant plasmid (pCoPDT) produced a 161 kDa protein that was recognized by antibodies specific to the three toxins. The expression of the PDT protein was inducible by isopropyl-β-D-thio-galactoside but the total amount of protein produced was relatively low. Attempts to improve the protein yield by expression in an *E. coli* strain (Rosetta-gami 2) that could alleviate rare-codon usage bias and by supplementation of the growth media with amino acids deemed to be a limiting factor in translation were not successful. The PDT protein remained in the insoluble fraction when the recombinant *E. coli* was grown at 37 °C but the protein became soluble when the bacteria were grown at 22 °C. The PDT protein was isolated via affinity chromatography on a NiCAM column. The protein was associated with five other proteins via disulfide bonds and non-covalent interactions. Following treatment with β-mercaptoethanol, the PDT fusion was purified to homogeneity by preparative polyacrylamide gel electrophoresis with a yield of 45 μg/L of culture. Antisera generated against the purified PDT protein recognized the native toxins indicating that some, if not all, of the native epitopes were conserved.

Keywords: Pertussis–diphtheria–tetanus toxin; Soluble; Recombinant; Expression

Pertussis toxin (PT) is an AB toxin produced by *Bordetella pertussis* with the A-protomer (S1 subunit) as the toxic subunit and B-oligomer as the pentamer that binds to cell receptors. The S1 subunit is composed of 234 amino acids and is immunodominant. Antibodies against S1 subunit have been shown to neutralize pertussis toxin *in vitro* and protect mice from aerosol *B. pertussis* infection [1,2]. The B oligomer is composed of one subunit each of S2, S3, and S5 and two subunits of S4. S2 and S3 mediate adherence of the toxin to host cells. Similar to S1 subunit, antibodies against B-oligomer or S2 and S3 subunit confer protection against *B. pertussis* infection in animal models, but are less effective than antibodies to S1 [2].

Diphtheria toxin (DT)[2] produced by *Corynebacterium diphtheriae* is a secreted 535 amino acids protein and is responsible for the symptoms of diphtheria. Following synthesis, DT is proteolytically cleaved into two fragments, A

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[2] *Abbreviations used:* DT, diphtheria toxin; TT, tetanus toxin; PT, pertussis toxin; FHA, filamentous hemagglutinin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PDT, pertussis–diphtheria–tetanus toxin; TTC, tetanus toxin fragment C; PCR, polymerase chain reaction.

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and B [3]. The catalytic domain is located on fragment A and the receptor and translocation domains on fragment B. Fragment B is responsible for DT binding to specific cell surface receptors and translocation of fragment A into the cytosol. Fragment A catalyzes the ADP-ribosylation of elongation factor-2 resulting in inhibition of protein synthesis and cell death. Expression of full-length and fragments of DT has been described in *Escherichia coli* [4], *Salmonella enterica* [5], *Staphylococcus carnosus* [6], *Mycobacterium bovis* [7], and recently in *Streptococcus mutans* and *Streptococcus gordonii* from our laboratories [8,9].

Tetanus toxin (TT) is a 150 kDa protein produced by *Clostridium tetani* as a single polypeptide and subsequently cleaved into a 100 kDa heavy (B chain) and a 50 kDa light (A chain) chain linked by two disulfide bonds. The carboxyl-terminal portion (TTC, 50 kDa) of the heavy chain binds to GT1b gangliosides on neural cells. The light chain, a zinc endopeptidase, is then internalized and transported to the central nervous system and released in the presynaptic nerve terminals. TT acts by blocking the release of neurotransmitters for inhibitory synapses, thus causing excitatory circuits to be unregulated giving rise to tetanic spasm. Immune protection against tetanus is mediated by toxin-neutralizing antibodies. TTC has been cloned in *Salmonella typhi* [10], *E. coli* [11], *S. gordonii* [12], and baculovirus [13]. The recombinant TTC is non-toxic and capable of inducing toxin-neutralizing antibodies.

DT and TT are well-characterized antigens currently used in licensed diphtheria–tetanus–acellular pertussis (DTaP) vaccines. In these vaccines, the pertussis antigens included are a combination of PT, filamentous hemagglutinin (FHA), pertactin, and fimbriae. Vaccines containing one or more of PT and FHA have a demonstrated efficacy very similar to that containing all four pertussis antigens. Although the efficacy of the DTaP vaccines is well documented, the vaccines are expensive to produce because the antigens are derived from three different bacteria. Hence, if antigens are engineered to be produced by one organism, vaccine production should be simplified and the cost of production be lowered. In this study, we have created a fusion protein containing the N-terminal 179 amino acid fragment of the S1 subunit of PT, that was shown to be protective in an animal infection model [14], the full-length genetically detoxified DT, and the 50 kDa tetanus toxin fragment C (TTC), which has also been shown to confer protection against toxin challenge in animal models [11–13]. The expression and purification of the fusion protein is described. The work represent a first step toward demonstrating a proof-of-principle that such an approach could be explored to generate a new generation of DTaP vaccines.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli XL1-blue (Stratagene, La Jolla, CA) was used for general cloning and maintenance of plasmids. *E. coli* Rosetta-gami 2 (Novagen, Madison, WI) was used as a host for protein production. This strain of *E. coli* harbored pRARE2 which carried seven rare-codon tRNA genes for overcoming codon usage bias. In addition, the glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) genes in this strain were inactivated to facilitate cytoplasmic disulfide bond formation. The bacteria were grown aerobically with shaking at 200 rpm in media and temperatures as described below. Antibiotics when needed were added to the medium at 100 μg/mL ampicillin, 10 μg/mL tetracycline, 70 μg/mL kanamycin, 50 μg/mL streptomycin, and 34 μg/mL chloramphenicol.

Construction of the trivalent pertussis–diphtheria–tetanus toxin (PDT) fusion protein

The construction of the PDT fusion protein gene is depicted in Fig. 1A. The 1.4 kb *Sma*I–*Kpn*I DNA fragment carrying the DNA coding for the N-terminal 179 amino acid fragment of pertussis toxin S1 subunit was obtained from pPTS1 [15] and cloned into the *Nru*I–*Kpn*I sites of pRJMI [15]. The cloning created pPT2S, which carried two copies of the S1 fragment in tandem fused to the N-terminal portion of SpaP, a major surface protein of *Streptococcus mutans* [16]. The reason for cloning two copies of S1 in tandem was because the construct has been shown to confer protection in animal infection studies [14]. The DNA coding for the full-length non-toxic DT was isolated as a 1.6 kb *Eco*RV–*Xba*I fragment from pDTX1 [8] and cloned into the *Nru*I–*Xba*I sites on pPT2S. The resulting plasmid was named pSS1. In this process, the *Cla*I–*Nru*I fragment containing the C-terminal amino acids required for the high affinity binding of S1 subunit to transducins [17] has been omitted and the remaining N-terminal 179 amino acid sequence of S1 fragment was non-toxic [18]. The 1.4 kb DNA coding for TTC was amplified from pSS1261 [19] by polymerase chain reaction (PCR) using primers SL203 (ACGATATCAAAAATCTGGATTGTTTGG, *Eco*RV site underlined), and SL204 (GCTCTAGATCCCGGGAT CATTTGTCCATCCTTC, *Sma*I and *Xba*I sites underlined, stop codon in bold) and *Taq* DNA polymerase. The PCR reaction consisted of 30 cycles of denaturation (1 min, 94 °C), annealing (1 min, 50 °C), and extension (2 min, 72 °C). The PCR product was restricted with *Eco*RV and *Xba*I and cloned into the same sites on pSMI/II [16] creating pTTC1. The 1.4 kb *Eco*RV–*Bam*HI fragment from pTTC1 was subsequently cloned onto the *Sma*I–*Bam*HI sites of pSS1 giving pPDT1. The cloning put the tandem S1 gene in frame to DT followed by TTC and fused to the N-terminus of SpaP creating a fusion protein of 1880 amino acids (Fig. 1C).

To facilitate the expression and subsequent protein purification, the PDT fusion gene was subcloned into pComb3X [20]. The fusion gene was amplified from pPDT1 by PCR using primers SL296 (TAGGCCGGCC TGGCCATCATTTGTCCATCCTT C, *Sfi*I site underlined) and SL332 (GGGCCCAGGCGGCCAAGGCTT

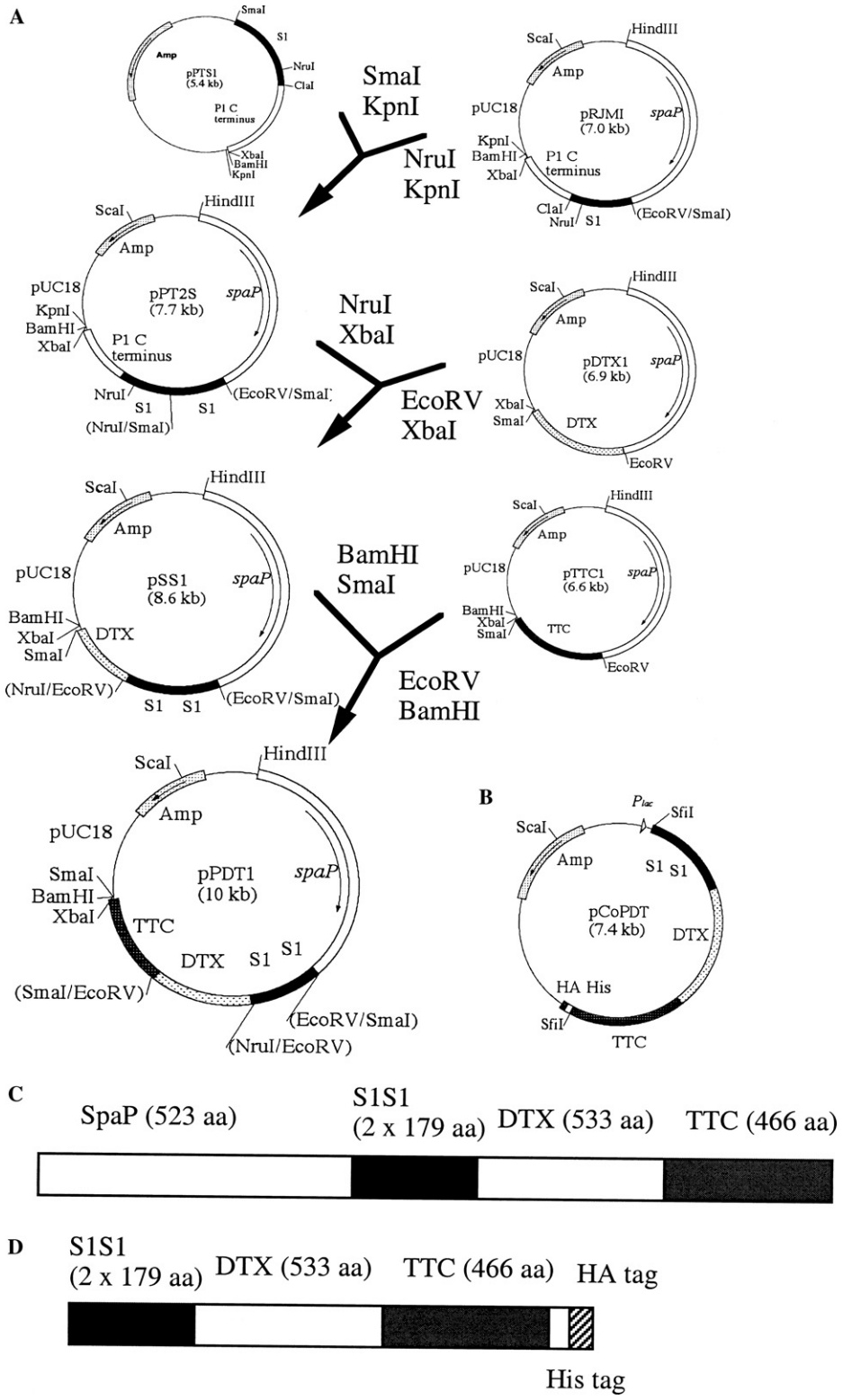

Fig. 1. Construction of the trivalent pertussis-diphtheria-tetanus toxin fusion gene in pPDT1 (A) and pCoPDT (B). The predicted fusion protein encoded by pPDT1 (C) and pCoPDT (D) is also shown. aa, amino acids.

CTGCTGT GGATG, *Sfi*I site underlined) and *Taq* DNA polymerase. The PCR reaction contained 30 cycles of denaturation (30 s, 94 °C), annealing (30 s, 50 °C), and extension (4.5 min, 72 °C). The 4 kb PCR product was digested with *Sfi*I and cloned into the same sites on pComb3X. The resulting plasmid was named pCoPDT (Fig. 1B). The cloning put the fusion gene under the control of the *lacZ* promoter and added a histidine tag and a hemagglutinin (HA) tag to the C-terminus (Fig. 1D).

SDS–PAGE and Western immunoblotting

Protein samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the buffer system of Laemmli [21], unless stated otherwise. Proteins were transferred to nitrocellulose membranes according to the method of Towbin et al. [22] and detected with either the anti-S1 monoclonal antibody A4 (1/2000, [2]), the mouse anti-DT polyclonal antibody (1/2000, [9]), the mouse anti-TT polyclonal antibody (1/2000), or the anti-HA monoclonal antibody (1/5000, Sigma–Aldrich Chemical, Oakville, ON) and the alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1/20,000, Sigma–Aldrich).

Optimization of the PDT fusion protein expression

To determine the best medium for the expression of the PDT fusion protein, the recombinant *E. coli* was grown in Luria-Bertani broth [LB, 1% tryptone, 0.5% yeast extract, and 1% NaCl (w/v)] and Super Broth [SB, 1% Mops, 3% tryptone, and 2% yeast extract (w/v)] with and without the supplementation of 450 μg/mL each of tyrosine, aspartic acid, asparagine, lysine, proline, and serine. The inclusion of these six amino acids in the media was to increase their availability because analysis of the deduced amino acid composition of PDT revealed they were present in much higher proportions than the authentic *E. coli* proteins. The media (2 mL containing 0.2% glucose) were inoculated with 40 μL of a 16 h culture and incubated at 37 °C. After 4 h, isopropyl-β-D-thio-galactoside (IPTG) was added to a final concentration of 0.1 mM and the cultures were further incubated for 12 h. At the end of growth, aliquots of the culture representing equivalent cell density was centrifuged and the cells were boiled in 200 μL of SDS–PAGE sample buffer for 10 min and the clear lysates were analyzed by Western blotting to detect the fusion protein using the anti-HA monoclonal antibody as the probe.

To examine the inducibility of the fusion protein, *E. coli* Rosetta-gami 2 (pCoPDT) was grown in SB media and induced with varying concentrations of IPTG as above. Following growth, equivalent amount of cells were lysed by boiling with SDS–PAGE sample buffer and the fusion protein was detected by the anti-S1 monoclonal antibody by Western immunoblotting,

Localization of the PDT fusion protein

Two 50 mL SB containing 0.2% (w/v) glucose were each inoculated with 1 mL of a 16 h culture of *E. coli* Rosetta-gami 2 (pCoPDT) grown at 37 °C. The cultures were incubated at 37 °C for 4 h at which time IPTG was added to 0.1 mM. One of the cultures was further incubated at 37 °C for 12 h and the other culture was incubated at 22 °C for the same length of time. At the end of growth, the cultures were centrifuged at 10,000*g* and 4 °C for 15 min. One mL of the culture supernatant was mixed with 0.25 mL 20% (w/v) tricholoroacetic acid and the precipitated proteins were resuspended in 0.1 mL SDS–PAGE sample buffer as the extracellular fraction. The cells were resuspended in 5 mL of NiCAM wash buffer (50 mM $Na_2HPO_4{\cdot}H_2O$, 300 mM NaCl, 5 mM imidazole, pH 8.0) and disrupted by sonication (10×20 s bursts at 60 amplitude separated by 10 s cooling on ice, Vibra cell™, Sonics and Materials Inc. Danbury, CT, USA). The sonicate was centrifuged at 17,500*g* for 20 min and the supernatant saved as the soluble fraction. The pellet was extracted with 1 mL of 0. 25% (w/v)Tween 20 and 1 mM EDTA, pH 8.0, at room temperature for 30 min. The mixture was centrifuged, the pellet extracted two more times, and the supernatant pooled as membrane-bound proteins. The final pellet representing the insoluble fraction was subsequently treated with 1 mL of 8 M urea in 100 mM $NaH_2PO_4{\cdot}H_2O$, pH 9.0. Aliquots of the fractions (10 μL of extracellular fraction, 4 μL of soluble fraction, 5 μL of membrane-bound proteins, and 2 μL of insoluble fraction representing roughly 100, 40, 83, and 100 μL of the original culture, respectively) were analyzed for the PDT fusion protein by Western immunoblotting using the anti-HA monoclonal antibody as the probe.

Partial purification of PDT by NiCAM column chromatography

Cells from two 1-litre cultures of *E. coli* Rosetta-gami 2 (pCoPDT) grown in SB at 22 °C with IPTG induction were harvested by centrifugation and resuspended in 10 mL of NiCAM wash buffer (pH 8) and sonicated. The soluble fraction was obtained by centrifugation and applied to a 4 mL NiCAM column (Sigma–Aldrich), which had been equilibrated in the NiCAM wash buffer. The column was washed with 20 mL of NiCAM wash buffer (pH 8), followed by 20 mL of NiCAM wash buffer (pH 4.5). The bound PDT protein was eluted with 20 mL of 250 mM imidazole in NiCAM wash buffer (pH 8).

The eluted PDT was mixed with denaturing and reducing PAGE sample buffer (with SDS and β-mercaptoethanol), non-reducing sample buffer (with SDS but no β-mercaptoethanol), non-denaturing sample buffer (with β-mercaptoethanol but no SDS), or non-denaturing and non-reducing sample buffer (no SDS and β-mercaptoethanol). The samples were either boiled for 5 min or not heated and separated by electrophoresion a 7.5% SDS–PAGE gel of Laemmli [21].

Purification of PDT by preparative polyacrylamide gel electrophoresis

The eluate containing the PDT protein from the NiCAM column was concentrated by ultrafiltration and incubated with SDS-free reducing sample buffer at room temperature for 15 min and applied to preparative 7.5% SDS–PAGE gels. Following electrophoresis, strips were excised from the edges of the gels and stained with Coomassie blue R-250 to reveal the position of the PDT

protein. Sections of the unstained gels containing the PDT protein were then excised and put in a dialysis bag containing 4 mL of SDS-free electrode buffer of Lammeli [21]. The protein in the gel slices was electroeluted in a horizontal DNA sub-cell containing the SDS-free electrode buffer under a constant voltage of 110 V at 4 °C for 4 h. Following electroelution, the polarity of the electric filed was reversed for 1 min and the content in the dialysis bag was recovered. The concentration of the electroeluted protein was estimated by densitometry in comparison to bovine serum albumin standards on a Coomassie blue-stained gel using the Image J program (NIH, Bestheda, MD).

Immunogenicity of the PDT fusion protein

The purified PDT fusion protein was incubated with 2% (w/v) aluminum hydroxide gel (Sigma–Aldrich) at room temperature for 18 h. After incubation, the mixture was centrifuged for 10 s and the supernatant was analyzed for proteins by SDS–PAGE and showed that >99% of the fusion protein had been adsorbed. The loose pellet containing the alum-adsorded fusion protein was resuspended in phosphate-buffered saline (10 μg/dose) and injected subcutaenously in two BALB/c mice (female, 6-week-old) on days 1, 14, and 21. Two additional mice were similarly immunized with a commercial DTaP vaccine (Quadracel, Sanofi Pasteur, Toronto, ON). The dosage for the DTaP group was 40 μL containing 1.6 μg each of FHA and pertussis toxoid, 0.4 μg fimbriae, and 0.24 μg pertactin. The animals were euthanized on day 26. Sera were obtained at day 0 and at euthanasia.

Results

Construction of the PDT fusion protein

PDT was constructed by ligating DNA coding for the respective fragments into a single 4.0 kb sequence (Fig. 1A). The initial construct was a fusion to the major surface protein SpaP and under the control of the *spaP* promoter (Fig. 1C). The correct construct was verified by restriction endonuclease analysis (data not shown). *E. coli* XL1-blue carrying pPDT1 was shown to express a 200 kDa protein band and three smaller proteins recognized by the anti-S1 monoclonal antibody, the anti-DT polyclonal antibody, and the anti-TT polyclonal antibody indicating correct fusion has been made (Fig. 2A). The 200 kDa protein was the expected full-length protein while the three smaller proteins were presumably degradation products. These protein bands were absent from the parent *E. coli* lysate. Because the expression of the fusion protein under the *spaP* promoter was relatively low and the lack of a convenient means to purify the fusion protein, the fusion protein gene was subcloned into pComb3X. The cloning added a histidine tag and hemagglutinin (HA) peptide tag to the C-terminus of the fusion protein (Fig. 1D). In addition, the expression of the fusion protein was controlled by the

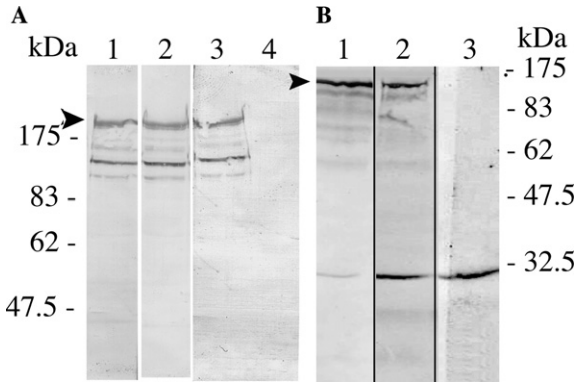

Fig. 2. Western immunoblotting of recombinant *E. coli* expressing the trivalent pertussis–diphtheria–tetanus toxin fusion protein. (A) *E. coli* XL1-blue carrying pPDT1. The PDT fusion protein in the *E. coli* lysates was detected by the anti-S1 monoclonal antibody (lane 1), the mouse anti-DT polyclonal antibody (lane 2), and the mouse anti-TT polyclonal antibody (lane 3). Lane 4 is the parent *E. coli* XL1-blue lysate probed with anti-TT antibody. (B) *E. coli* Rosetta-gami 2 carrying pCoPDT. The PDT fusion protein was detected by the anti-HA antibody (lane 1) and anti-S1 antibody (lane 2). Lane 3 is *E. coli* Rosetta-gami 2 lysate probed with the anti-S1 antibody as a control. Arrow heads indicate the full-length fusion protein.

IPTG-inducible *lacZ* promoter. The predicted fusion protein consisted of 1394 amino acids of S1S1DTTTC, six amino acids of histidine tag, and 10 amino acids of HA tag. Western immunoblotting of *E. coli* lysates carrying pCoPDT showed a 161 kDa protein band that was recognized by the anti-S1 monoclonal antibody and the anti-HA antibody indicating the fusion gene has been successfully subcloned (Fig. 2B).

Optimization of PDT fusion protein expression

To investigate the best medium for protein expression, the recombinant *E. coli* was grown in LB and SB with and without supplementation of tyrosine, aspartic acid, asparagine, lysine, proline, and serine. The results showed that the fusion protein was expressed when the bacteria were grown in SB but not in LB (Fig. 3A). The level of the fusion protein expressed in SB was similar with and without amino acid supplementation. Both *E. coli* Rosetta-gami 2 and *E. coli* XL1-blue cells produced similar amount of the PDT fusion protein.

The expression of the PDT protein could be induced by IPTG. As shown in Fig. 3B, in the absence of IPTG, only a low level of PDT was produced. The level of PDT produced increased with increasing concentrations of IPTG and reached a plateau at 0.1 mM IPTG.

The cellular location of the PDT fusion protein in *E. coli* was investigated. The results showed that when the cells were grown at 37 °C, the majority of the PDT protein was associated with the insoluble fraction (Fig. 4). When the culture was grown at 22 °C, a significant amount of the PDT protein remained in the insoluble fraction, but the protein was also found in the soluble fraction and membrane-bound fraction. A trace of the fusion protein was also found in the extracellular fraction.

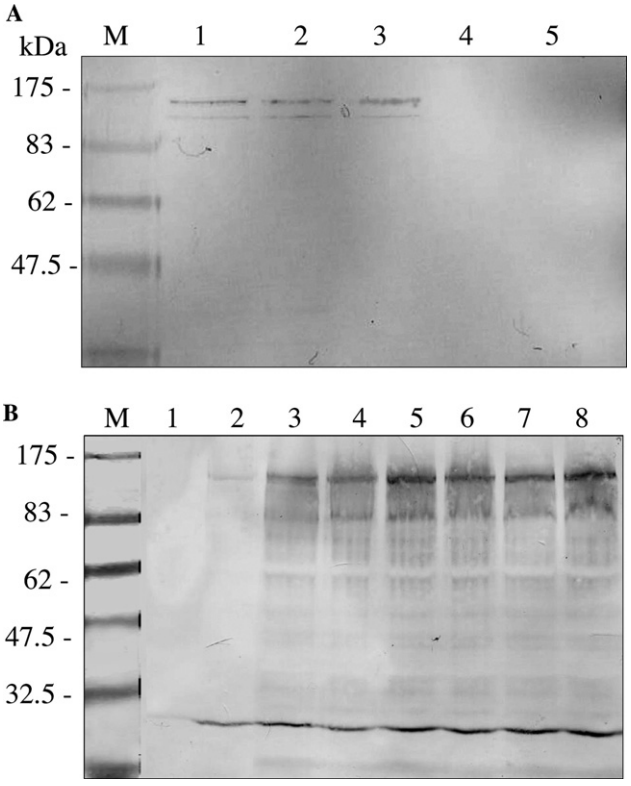

Fig. 3. Effects of media, amino acid supplementation, and IPTG concentrations on the expression of the PDT fusion protein by *E. coli*. (A) Effects of media and amino acid supplementation. Lanes 1, *E. coli* XL1-blue (pCoPDT) in SB medium; 2, *E. coli* Rosetta-gami 2 (pCoPDT) in SB medium; 3, *E. coli* Rosetta-gami 2 (pCoPDT) in SB medium supplemented with six amino acids; 4, *E. coli* Rosetta-gami 2 (pCoPDT) in LB medium; 5, *E. coli* Rosetta-gami 2 (pCoDTP) in LB medium supplemented with six amino acids. (B) *E. coli* Rosetta-gami 2 (pCoPDT) induced by IPTG. Lanes 1, parent *E. coli* Rosetta-gami 2 cell lysate; 2, 0 mM IPTG; 3, 0.025 mM IPTG; 4, 0.05 mM IPTG; 5, 0.1 mM IPTG; 6, 0.2 mM IPTG; 7, 0.4 mM IPTG; 8, 0.8 mM IPTG. In both (A and B), lane M is the prestained protein markers.

Purification of recombinant protein

From the results above, *E. coli* Rosetta-gami 2 (pCoPDT) was grown at 22 °C with 0.1 mM IPTG induction and the cell lystae was used as the source for PDT isolation by affinity chromatography on a NiCAM column. The results from a number of preliminary experiments demonstrated that a substantial amount of contaminating proteins remained bound to the column following washes with the NiCAM wash buffer (pH 8) and these proteins could be eliminated by washing the column with a pH 4.5 NiCAM buffer (Fig. 5A). The PDT fusion protein was eluted in 250 mM imidazole with five other proteins. Attempts to separate the PDT protein from these proteins by altering the concentration of imidazole were unsuccessful. The eluted PDT protein was subjected to DEAE-Sepharose, hydroxylapatite, phenyl-Sepharose, or gel filtration column chromatography and the separation of PDT from the five proteins was unsuccessful indicating that these proteins were closely associated with each other (data not shown). The relative amount of each of these proteins were estimated by densitometry and summarized in Table 1.

The eluted proteins from the NiCAM column were analyzed by PAGE under various conditions (Fig. 5B). The results showed that in the absence of β-mercaptoethanol, PDT and four other proteins, except the 60 kDa protein, did not enter the gel and remained in the sample well (Fig. 5B, lanes 3 and 4). In the presence of β-mercaptoethanol the proteins were separated (Fig. 5B, lane 6). SDS and heat had no apparent effects on the separation of PDT from the contaminating proteins.

Given the above results, preparative PAGE was employed to purify PDT. As shown in Fig. 6A, the PDT protein isolated by this means was a single protein with an expected molecular mass of 161,000. The yield of the

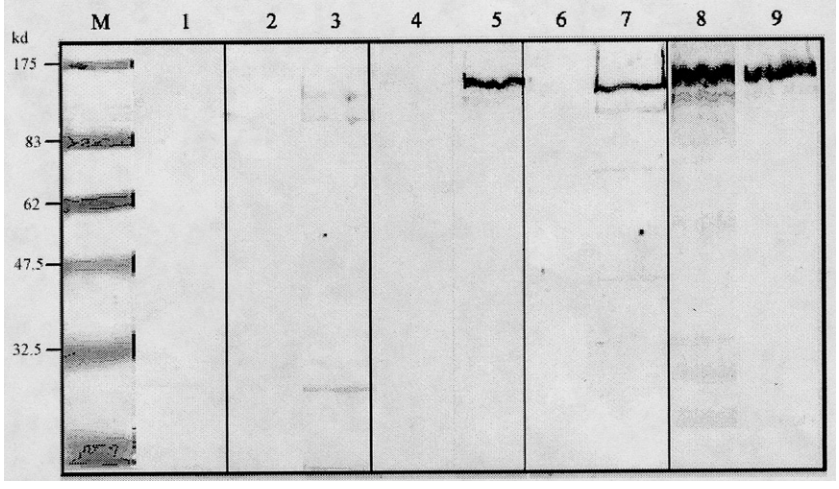

Fig. 4. Effects of culture temperature on the localization of PDT in *E. coli* Rosetta-gami 2 (pCoPDT). M, prestained protein markers. Lanes 1, parent Rosetta-gami 2 cell lysate as a control; 2, extracellular fraction from 37 °C culture; 3, extracellular fraction from 22 °C culture; 4, soluble fraction from 37 °C culture; 5, soluble fraction from 22 °C culture; 6, membrane-bound proteins from 37 °C culture; 7, membrane-bound proteins from 22 °C; 8, insoluble fraction from 37 °C culture; 9, insoluble fraction from 22 °C culture. The PDT fusion protein was detected with the anti-HA antibody.

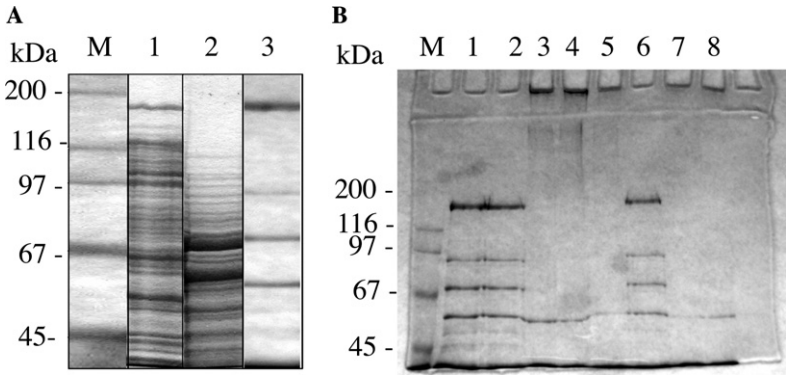

Fig. 5. Partial purification of PDT. (A) SDS–PAGE of protein samples from NiCAM column chromatography. M, high molecular mass protein markers; lanes 1, crude cell sonicate; 2, pH 4.5 wash; 3, 250 mM imidazole eluate containing the PDT fusion protein. (B) Analysis of the partially purified PDT proteins from the NiCAM column by polyacrylamide gel electrophoresis. M, high molecular mass protein markers. Samples were prepared in denaturing and reducing sample buffer (lanes 1 and 2), non-reducing sample buffer (lanes 3 and 4), non-denaturing sample buffer (lanes 5 and 6), and non-denaturing and non-reducing sample buffer (lanes 7 and 8). The samples in lanes with odd and even numbers were boiled for 5 min or not heated, respectively. In both (A and B), the proteins were stained with Coomassie blue R-250.

Table 1
The relative amount of PDT to the co-isolated proteins obtained from NiCAM column chromatography as estimated by densitometry

Proteins (estimated MW, Dalton)	Weight ratio	Molar ratio	Linkage bond
160990 (PDT)	1	1	—
95576	0.242	0.407	Disulfide
72377	0.486	1.080	Disulfide
59663	0.560	1.649	Non-covalent
47011	0.0462	0.158	Disulfide
41181	0.0914	0.357	Disulfide

PDT protein was ca. 45 μg/L of culture representing 87-fold of purification (Table 2). Western immunoblotting showed that the purified PDT protein was recognized by the anti-S1, anti-TT, anti-DT, and anti-HA antibodies (Fig. 6B). These results indicate that the purified protein was the PDT fusion protein.

Antisera generated against the purified PDT protein recognized the native toxins

The purified PDT was used to immunize mice and antisera generated were tested for recognition of the native antigens. As shown in Fig. 7, the anti-PDT antisera recognized a ca. 28 kDa band from PT and a 60 kDa band from DT. These bands were similarly recognized by sera raised against a commercial DTaP vaccine. The anti-PDT sera recognized a 83 kDa band from TT which was also recognized by the sera generated from the commercial vaccine. The latter also recognized a smaller band of ca. 50 kDa. None of these bands was observed in blots reacted with the pre-immune sera.

Discussion

In this study, the PT S1 fragment, the full-length non-toxic DT, and fragment C of TT were genetically linked as a single fusion protein. The correct in-frame fusion was demonstrated by results from Western immunoblotting showing the recognition of the fusion protein by specific antibodies to the toxins.

Expression studies clearly showed that the PDT fusion protein was produced by the recombinant *E. coli*. The expression level was similar between XL1-blue and Rosetta-gami 2 despite the latter strain having the ability to

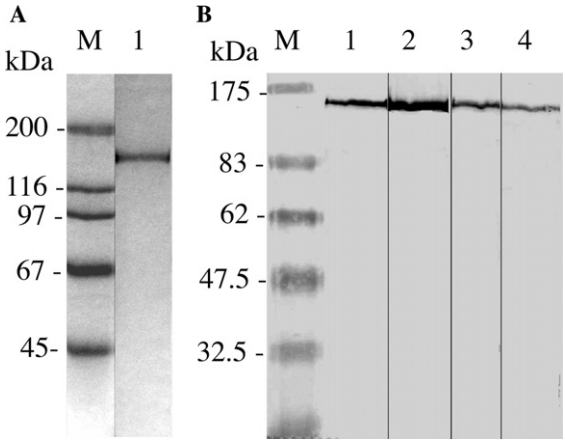

Fig. 6. SDS–PAGE and Western immunoblotting of the purified PDT protein. (A) SDS–PAGE. M, high molecular mass protein markers; lane 1, Purified PDT. (B) Western blots. M, prestained protein markers; lanes 1, monoclonal anti-HA antibody; 2, monoclonal anti-S1 antibody; 3, anti-DT antibody; 4, anti-TT antibody.

Table 2
Purification of the PDT fusion protein from *E. coli* Rosetta-gami 2 harboring pCoPDT

Purification step	Total PDT protein (μg)	Purification fold	Yield
Crude extract	220	1	100
NiCAM chromatography	176	36	80
PAGE	90	87	41

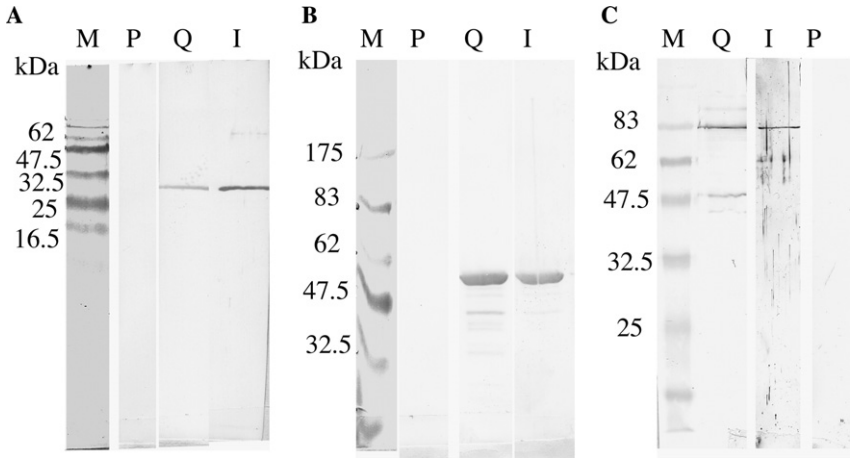

Fig. 7. Recognition of native toxins by antisera generated against the PDT fusion protein. (A) One μg of PT (List Biological Laboratories, Campbell, CA) separated on a 15% SDS–PAGE gel; (B) one μg of DT (list biological laboratory) separated on a 7.5% SDS–PAGE gel, and (C) 0.3 μg of TT (Sigma–Aldrich) separated on 10% SDS–PAGE gel. M, pre-stained protein markers; P, preimmune sera, Q, sera from mice immunized with a commercial DTaP vaccine; I, sera from mice immunized with the PDT fusion protein.

alleviate rare-codon usage bias. The PDT protein contained 127 rare-codons (13 CUA (leu), 36 AUA (ile), 14 CCC (pro), 3 CGA (arg), 7 CGG (arg), 11 AGA (arg), 14 AGG (arg), and 29 GGA (gly)) representing 9.1% of the total 1394 amino acids. The results suggest that the presence of these rare-codons may not have played a role in the low expression of the fusion protein. However, the culture medium did have an effect on PDT expression. The PDT protein was produced when the bacteria were grown in SB but not in LB. Since LB and SB are essentially the same media except for additional nutrients in the latter suggesting that the extra nutrients are needed for PDT production. The addition of six amino acids, which are deemed to be present in high proportion in the predicted amino acid sequence of the fusion protein and may create a bottle-neck in translation, had no apparent effect on the expression of PDT. Mahishi and Rawal [23] using such an approach were able to increase the expression of polyhydroxybutyrate in *E. coli*; however, the approach did not work in this case.

The expression of PDT could be induced by IPTG; however, the actual amount of protein produced was still relatively low. The total amount of PDT produced was estimated to be 110 μg/L of culture. The yield of the purified PDT protein is ca. 45 μg/L of culture from the soluble fraction. The reason for the low expression is not clear. As discussed above, the presence of rare-codons, abundance of the six amino acids, and *E. coli* host strains do not appear to be the reason. It is possible that high level expression of PDT is toxic to the cell and therefore high level of expression cannot be achieved.

It is interesting to note that the cellular location of the PDT protein is affected by culture temperature. At 37 °C, the PDT protein is exclusively found in the insoluble fraction. At 22 °C, about half of the protein is soluble and another half remains membrane-bound or insoluble. The trace amount of the protein found in the spent culture medium is likely due to limited cell lysis or from membrane blebbing. Similar findings were reported for the expression of the *E. coli* sigma 70 polypeptide [24], phage K11 RNA polymerase [25], murine terminal deoxynucleotidyl transferase [26], and *Plasmodium* histo-aspartic protease [27].

The PDT protein present in the soluble fraction is closely associated with five other proteins. Four of the five proteins appear to be associated with PDT via disulfide bonds (Table 1). The 60 kDa protein seems to be associated with PDT by non-covalent interactions that can be dissociated by SDS at ambient temperature. The identity of these co-isolated proteins is not known. Molecular chaperones, such as the Hsp proteins, are known to promote protein folding [28] and therefore could be associated with PDT. Western immunoblotting using the anti-*E. coli* Hsp60 antibody as the probe did not show reaction with any of the five co-isolated proteins indicating these are not Hsp60 (data not shown). The simultaneous association of four different proteins to PDT via disulfide bonds is intriguing. The relatively more oxidized environment in the cytoplasm of Rosetta-gami 2 resulting from the mutation in the *gor* and *trxB* genes and the presence of 10 cysteine residues in the PDT sequence may have facilitated the interaction.

Antisera generated against the PDT protein recognized the native PT, DT, and TT further indicating that correct fusion was made. More importantly, the results indicate that some, if not all, of the immunogenic epitopes are conserved in the recombinant protein. These results show that the approach taken in this study to generate a new DTaP vaccine antigen is promising. Further experiments are required to assess the quality of the antibodies and the protective efficacy of the fusion protein in animal models.

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